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ORIGINAL PAPER

Effects of N-starvation and C-source on *Bradyrhizobium japonicum* exopolysaccharide production and composition, and bacterial infectivity to soybean roots

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Abstract The exopolysaccharide (EPS) is an extracellular molecule that in *Bradyrhizobium japonicum* affects bacterial efficiency to nodulate soybean. Culture conditions such as N availability, type of C-source, or culture age can modify the amount and composition of EPS. To better understand the relationship among these conditions for EPS production, we analyzed their influence on EPS in *B. japonicum* USDA 110 and its derived mutant ΔP22. This mutant has a deletion including the 3′ region of *exoP*, *exoT*, and the 5′ region of *exoB*, and produces a shorter EPS devoid of galactose. The studies were carried out in minimal media with the N-source at starving or sufficient levels, and mannitol or malate as the only C-source. Under N-starvation there was a net EPS accumulation, the levels being similar in the wild type and the mutant with malate as the C-source. By contrast, the amount of EPS diminished in N-sufficient conditions, being poyhydroxybutyrate accumulated with culture age. Hexoses composition was the same in both N-situations, either with mannitol or malate as the only C-source, in contrast to previous observations made with different strains. This result suggests that the change in EPS composition in response to the environment is not general in *B. japonicum*. The wild type EPS composition was 1 glucose:0.5 galactose:0.5 galacturonic acid:0.17 mannose. In ΔP22 the EPS had no galactose but had galacturonic acid, thus indicating that it was not produced from oxidation of UDP-galactose. Infectivity was lower in ΔP22 than in USDA 110. When the mutant infectivity was compared between N-starved or N-sufficient cultures, the N-starved were not less infective, despite the fact that the amounts of altered EPS produced by this mutant under N-starvation were higher than in N-sufficiency. Since this altered EPS does not bind soybean lectin, the interaction of EPS with this protein was not involved in increasing ΔP22 infectivity under N-starvation.

Keywords *Bradyrhizobium japonicum* · EPS · Nitrogen · Infectivity · Symbiosis · Soybean

Abbreviations

CPS	Capsular polysaccharide
DAI	Days after inoculation
EPS	Exopolysaccharide
Gal	Galactose
GalA	Galacturonic acid
Glc	Glucose
GlcA	Glucuronic acid
Mal	Malate
Man	Mannose
Mtl	Mannitol
PHB	Polyhydroxybutyrate
OD_{500}	Optical density at 500 nm
RDU	Relative distance unit

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RT Root tip
SBL Soybean lectin
SERH Smallest emergent root hairs

Introduction

Bradyrhizobium japonicum is a soil bacterium that induces the formation of root nodules in soybean, among other hosts. Inside the nodules, it fixes atmospheric N_2 in symbiosis with the plant, providing N to soybean crops in an environmentally sustainable process by comparison to the use of fertilizers (Panzieri et al. 2000; Patriarca et al. 2004). To ensure a significant amount of N contribution from symbiosis, soybeans are inoculated with high quality N_2-fixing strains, from which occupation of most of the root nodules is expected, even when indigenous or adapted compatible rhizobial populations are present in the soil. The efficiency of a given strain to rapidly infect the roots and occupy the root nodules is referred to as its infectivity (Bhuvaneswari et al. 1980). Therefore, the genes as well as the physiological and environmental conditions that could influence the infectivity of inoculated strains deserve special attention (Gage 2004).

Rhizobial surface polysaccharides are among the main factors that affect infectivity in diverse species and symbiotic interaction types, although the exact role of these polysaccharides is not fully understood (Fraysse et al. 2003). These polysaccharides include the lipopolysaccharide, the cyclic glucans, the capsular polysaccharide, and the exopolysaccharide. Even when different polysaccharides can partially complement structural defects in others (Hozbor et al. 2004), their amount, size, structure and composition are determinant for their symbiotic functions.

In *B. japonicum* the capsular polysaccharide (CPS) and the exopolysaccharide (EPS) have the same general structure and differ only by their location, which is the CPS at the cell surface, while the EPS is secreted outside the cell; therefore both are commonly referred to as EPS. Mort and Bauer (1982) established the structure of this polysaccharide as a polymer of a 4-(*O*-methyl or *O*-acetyl)-galactose (Gal) $\alpha1 \rightarrow 6$ glucose (Glc) $\alpha1 \rightarrow 3$ Glc $\beta1 \rightarrow 3$ 4-*O*-acetyl-galacturonic acid (GalA) $\alpha1 \rightarrow 3$ mannose (Man) pentasaccharide repeating unit. Repeating units are linked by Man $\alpha1 \rightarrow 3$ Glc bonds, thus joining the reducing end of one pentasaccharide to the non-reducing end of the next, leaving the substituted Gal residue to branch outside the main chain. The presence of *O*-methyl or *O*-acetyl substituents at the C-4 of this Gal residue depends on the physiological growth state of the bacteria: old, stationary phase cultures have *O*-methyl substituents while young, exponentially growing cultures have *O*-acetyl as a substituent (Mort and Bauer 1980). These changes correlate well with soybean lectin (SBL) binding to the rhizobia and soybean roots infectivity. Whiles *O*-methyl-Gal derivatives are poor SBL haptens, *O*-acetyl-Gal, as well as *N*-acetyl-Gal derivatives, are potent ones (Lis et al. 1970). As a parallel, old rhizobia have low SBL binding activity and soybean infectivity whereas young rhizobia have high performances in both activities (Mort and Bauer 1980; Bhuvaneswari et al. 1983; López-García et al. 2001).

Changes in *B. japonicum* EPS accumulation and composition were observed also in response to the cell nutritional environment and after nodule infection. Cultivation of rhizobia with mannitol (Mtl) as the sole C-source and under N-starving conditions was shown to enhance EPS accumulation even in old cultures, and the higher EPS content again correlated to higher SBL binding and infectivity (López-García et al. 2001). On the other hand, Karr et al. (2000) reported that the EPS composition in the *B. japonicum* strain 2143 changed according to the C-source used for growth. In particular, when grown on Mtl as the sole C-source, the EPS composition reflected the pentasaccharide repeating unit described above, while if grown in malate (Mal) as the sole C-source, the EPS composition was 100% unmodified Gal. Although Gal is also recognized by SBL, it is not such a strong hapten as Gal-acetylated derivatives (Lis et al. 1970). In agreement with the changes noted in EPS composition, Karr et al. (2000) observed that Mal-grown 2143 rhizobia displayed a poor binding of SBL; however, this strain had normal infectivity and nodulating ability in these conditions. Yet another different EPS, called nodule polysaccharide (NPS), is made into the root nodules by several *B. japonicum* strains. This NPS is composed of 1 Gal:3 rhamnose:1 2-*O*-methyl-GlcA (An et al. 1995) and its presence was correlated with the serogroups that were the most competitive for nodule occupation in the USA (Streeter et al. 1992).

Studies with mutant strains demonstrated that EPS is also a key determinant in suppressing plant defense. For this activity, the structure and composition of the EPS are critical but as of yet, it is unknown whether the wild type EPS masks some rhizobial plant defense elicitor, or altered EPS may act themselves as elicitors. *B. japonicum* ΔP22 is a USDA 110-derived mutant, which lacks UDP-Glc-4′-epimerase activity and produces low amounts of an EPS shorter than the wild

type and devoid of Gal (Parniske et al. 1993; Becker et al. 1998). This mutant nodulates later and triggers several plant defense responses such as glyceollin production in the root exudates and chitinase activity at the nodule cortex, which are not seen in plant roots invaded by the wild type (Parniske et al. 1994). Additionally, this strain was shown as being unable both to bind SBL and to nodulate transgenic *Lotus* plants expressing SBL, contrary to the wild-type *B. japonicum* (van Rhijn et al. 1998).

In our studies with N-limited rhizobia referred to above (López-García et al. 2001) we observed that N-limitation enhances SBL binding and rhizobial infectivity but we do not know whether a possible change in EPS composition is produced, as was observed with different C-sources (Karr et al. 2000) or inside nodules (An et al. 1995). Therefore, we were interested in assessing whether N-limitation induces changes in EPS composition either with Mtl or Mal as the sole C-source. Moreover, we compared the wild type USDA 110 with the EPS-defective, SBL non-binding strain ΔP22 in the above-mentioned conditions to further characterize the C flow to EPS as well as the role of EPS structure in relation with infectivity.

Methods

Rhizobial strains, growth and maintenance

Bradyrhizobium japonicum USDA 110 was obtained from the United States Department of Agriculture (Beltsville, USA). The strain ΔP22 (Sp^r, Km^r) was kindly provided by Peter Müller (Marburg, Germany). This mutant was genotypically and phenotypically characterized elsewhere (Parniske et al. 1993, 1994; Becker et al. 1998). It has a deletion that encompasses the 3′ region of *exoP*, the entire coding sequence of *exoT*, and the 5′ region of *exoB*. The first gene has homology to *Sinorhizobium meliloti exoP*, which is involved in the polysaccharide export, the second has homology to various transferases, and *exoB* encodes an UDP-Glc-4′-epimerase (Becker et al. 1998). Both strains were maintained in yeast extract-mannitol (Vincent 1970) with 30% v/v glycerol at –80°C. For routine use, cultures were propagated on yeast extract-mannitol agar plates supplemented with Congo Red and, in the case of ΔP22, 200 μg spectinomycin (Sp) ml^{-1} and 150 μg kanamycin (Km) ml^{-1} were added. These cultures were maintained at 4°C and renewed every 1 or 2 months. No more than four to five subculturing rounds in yeast extract-mannitol agar were done, after which new cultures were initiated from the frozen stocks. In this medium, the USDA 110 strain had smooth white colonies 3–5 mm in diameter after 10 days at 28°C, whereas ΔP22 had smaller, red colonies as expected from its defect in EPS production.

For experimental purposes a single colony of the indicated strain was grown in 10 ml liquid Götz minimal medium (Götz et al. 1982) that contained 27 mM Mtl as the sole C-source, the following salt's composition in mM: $(NH_4)_2SO_4$ 1.0; $MgSO_4$ 1.0; K_2HPO_4 6.1; KH_2PO_4 3.9; $CaCl_2$ 0.1; NaCl 0.1; Na_2MoO_4 0.01; $FeSO_4$ 0.001, the following vitamins at 0.02 mg l^{-1} each: biotin, thiamine–HCl, riboflavin, *p*-aminobenzoic acid, pyridoxine, without antibiotics, and the pH adjusted to 7.0 with 40 mM MOPS buffer (MOPS-Götz). After 1 week of growth in a rotary shaker at 28°C and 180 rpm, this culture was diluted 1:100 in fresh MOPS-Götz where the C-sources were 27 mM Mtl or 27 mM Mal. Each C-source was added to the C-free sterile media from filter-sterilized stock solutions in 40 mM MOPS, pH = 7.0. After 2 days of growth in these conditions, all cells were in the exponential growth phase (López-García et al. 2001). Then, the experimental cultures were started by diluting this culture in the appropriate volumes of test media to an optical density at 500 nm (OD_{500}) of 0.01, as recorded with a BioRad SmartSpec 3000 spectrophotometer. Test media were based on MOPS-Götz with the C- and N-sources modified as follows: Mtl N1 had 27 mM Mtl and 1 mM $(NH_4)_2SO_4$, Mtl N0 was the same without addition of $(NH_4)_2SO_4$, Mal N1 had 27 mM Mal and 1 mM $(NH_4)_2SO_4$, and Mal N0 was the same without addition of $(NH_4)_2SO_4$. The C-source concentrations were similar as those used by Karr et al. (2000). It was previously shown that N did not limit growth in Mtl N1, and the N-starved state in Mtl N0 was already characterized (López-García et al. 2001).

Total biomass was estimated from OD_{500} and viable bacteria from the number of colony-forming units (c.f.u.) in yeast extract-mannitol agar plates. It was previously determined that colony growth in yeast extract-mannitol agar is identical to that in Götz-agar.

Polysaccharide preparations

Three hundred milliliter cultures of *B. japonicum* USDA 110 or ΔP22 were centrifuged at 12,000*g* for 40 min. The cell pellets were reserved for protein and polyhydroxybutyrate (PHB) determinations. The EPS was precipitated from the supernatant with three volumes of ethanol 96% at –20°C and, after resuspension in 0.5 M NaCl, it was dialyzed against double-distilled water and lyophilized. This precipitate was finally resuspended in double-distilled water.

Analytical determinations

The cell pellets from the above fractionation were resuspended in phosphate buffer pH = 7.0 and separated in two fractions. For protein extraction, one of the fractions was disrupted by ultrasonic treatment with a Sanyo Soniprep 150 using three 10-s pulses at mean power. Following centrifugation, protein concentrations were determined as described (Bradford 1976) in the clear supernatants from sonicated cells. For PHB extraction, the other fraction was homogenized with sodium hypochloride as described (Cevallos et al. 1996) and PHB was determined as chrotonic acid in H_2SO_4 (Law and Slepecky 1961).

Polysaccharides were determined in culture supernatants with 0.2% w/v anthrone in 95% H_2SO_4, as described (Trevelyan et al. 1952), using glucose as standard. For HPAEC analysis of EPS composition, total hydrolysis was performed with 2 M trifluoroacetic acid at 120°C during 2 h. A DX-300 Dionex BioLC system with a pulse amperometric detector and a Carbopack PA-10 column was used. The following conditions were employed: (a) For neutral sugar analysis: an isocratic elution with 7% solution A (200 mM NaOH), 93% solution B (water). Flow: 1 ml min^{-1}; 2-deoxyglucose was used as internal standard. (b) For acidic sugars analysis: an isocratic elution with 25% solution A, 65% solution B and 10% solution C (1 M Na acetate). Flow: 1 ml min^{-1}; sialic acid was added as internal standard. A response factor for GalA analysis was determined. Averages from triplicate injections were calculated for each sample.

Plant experiments

Soybean cv Don Mario 4800 seeds were kindly provided by Alejandro Perticari (INTA-Castelar, Argentina). The seeds were surface sterilized by immersion in 96% ethanol for 5 s and then in 20% (v/v) commercial bleach for 10 min, followed by six sterile-distilled water washes. Seeds were germinated on aqueous agar (1.5% w/v) in darkness.

Nodulation profiles were obtained by inoculating 30 plants with the indicated concentrations of rhizobia $plant^{-1}$ in plastic growth pouches watered with a modified N-free Fåhraeus solution (Lodeiro et al. 2000). At the time of inoculation, the positions of the smallest emergent root hairs (SERH) and the root tip (RT) were marked on the surface of each plastic growth pouch. The distance between SERH and RT marks for each individual plant was taken as the relative distance unit (RDU) for that plant. After 20 days of growth in the greenhouse at 26°C/18°C day/night temperature, all nodule positions in the primary root of each plant were recorded in mm (± 0.5 mm) with respect to the corresponding RT mark. Nodule distances to RT were expressed in RDU to compensate for the different elongation rates and SERH/RT lengths among individual plants. Nodules appearing above the RT mark were given positive values, while those appearing below the RT mark received negative values (for details, see Bhuvaneswari et al. 1980).

Results

Bacterial growth

The growth of *B. japonicum* was analyzed in four culture conditions: *Mtl N1*, with mannitol as the C-source and N-sufficiency; *Mtl N0*, with mannitol as the C-source and N-starvation; *Mal N1*, with malate as the C-source and N-sufficiency, and *Mal N0*, with malate as the C-source and N-starvation. We previously characterized these physiological states of N-sufficiency and N-starvation by assessing the regulation of glutamine synthetase specific activity (López-García et al. 2001). In the latter report we also observed that raising $(NH_4)_2SO_4$ concentration to 10 mM gives no response in growth, thus demonstrating that N was not the limiting nutrient at 1 mM $(NH_4)_2SO_4$ in this minimal medium. Here we assume that the same conditions for N-sufficiency and N-starvation hold with Mal as the C-source.

All cultures were started with exponentially growing cells in the corresponding C-source and N-sufficiency. Furthermore, total biomass and viable cells either in N-sufficiency or N-starvation were recorded throughout 25 days with Mtl as the C-source, or 8 days with Mal as the C-source. Three independent experiments were performed with similar results.

In Fig. 1 we show the complete set of OD_{500} readings from one of these experiments. These results, together with plate counts (not shown) indicated that growth rates of both *B. japonicum* strains were significantly higher in Mal (doubling time: 12 h) than in Mtl (doubling time: 22 h) as described before (Karr et al. 2000). We also noted minor differences between the wild type and the mutant, being USDA 110 slightly faster than ΔP22 in each C-source. When N-starved conditions were employed, the exponential growth rate was the same but the stationary phase was reached earlier. Interestingly, the total OD_{500} and colony counts obtained with ΔP22 under N-starvation (Mtl N0 and Mal N0) were, in the three independent experiments, consistently higher than those obtained with

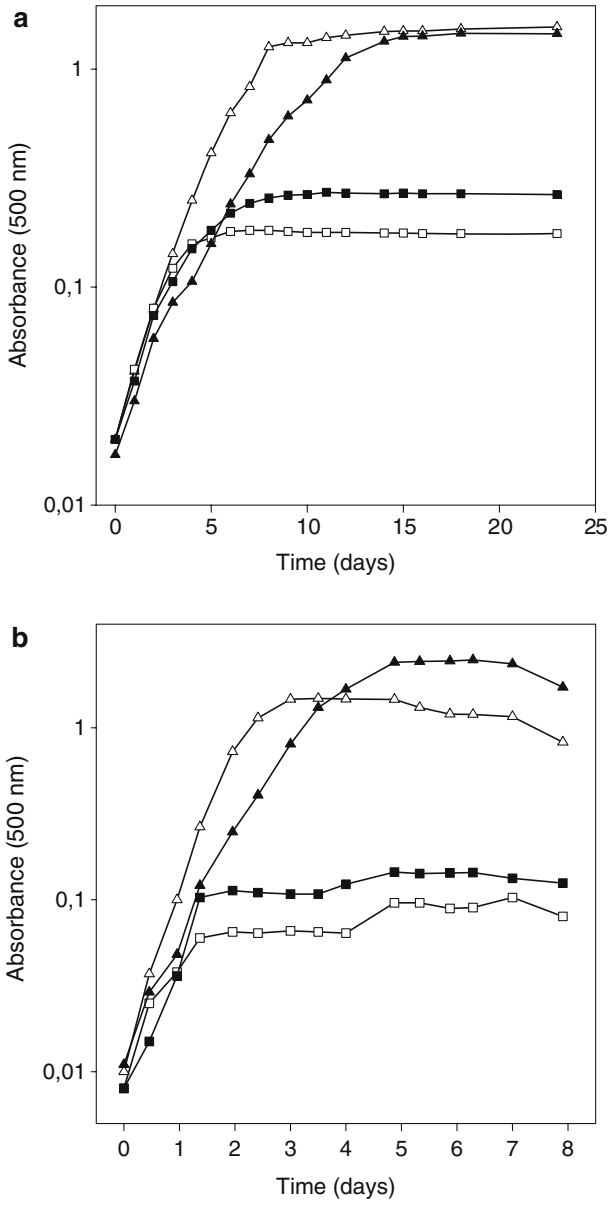

Fig. 1 Growth of *B. japonicum* in Götz-derived minimal media with different C and N composition. **a** 27 mM Mtl as C source. **b** 27 mM Mal as C source. In both plots, N-source was 1 mM $(NH_4)_2SO_4$ (*triangles*) or no N source addition (*squares*). Growth was measured as OD_{500}, with the strains USDA 110 (*white symbols*) or ΔP22 (*black symbols*). A complete set of OD_{500} readings, representative of three independent experiments

USDA 110 (Fig. 1). Once stationary phase was reached, no significant losses in viability were observed in any of the four conditions (not shown).

EPS accumulation and composition in the different culture media

We stated before that C is accumulated in different polymers when the wild type strain *B. japonicum* LP 3001, a Sp-resistant derivative from USDA 110, shifts from young and actively dividing rhizobia to old cultures, depending on whether stationary phase is reached after N-starvation or not. In the first case, EPS is accumulated in the stationary phase, whereas in a N-sufficient condition, the content of this polysaccharide diminishes, while PHB is accumulated instead (López-García et al. 2001).

Here we further investigated the effect of N-growth level with different C-sources on EPS accumulation in relation to total protein or PHB. Given the different growth rates observed for each C-source (Fig. 1), we decided to work with cultures of different ages as representative of young and old states. Young cultures were harvested at 2 and 5 days after inoculation (DAI) in Mal or Mtl media respectively, while old cultures were collected at seven and fourteen DAI with these respective C-sources. Since the stationary phase is reached earlier in N-starved cultures, the physiological growth state is not necessarily the same for a given chronological age in a N-starved or N-sufficient culture. On the other hand, we allowed the exponential growth phase to evolve long enough in the N-starved cultures to dissipate any influence of the previous N-sufficient state from the starter culture (López-García et al. 2001).

To assess the extent to which EPS and PHB compete as C-sinks, we compared the wild type with a mutant defective in EPS production. We chose ΔP22, derived from USDA 110 and already characterized elsewhere (Parniske et al. 1993, 1994; Becker et al. 1998). Biochemical analyses demonstrated that this mutant lacks UDP-Glc-4′-epimerase activity as well as the Leloir pathway, and produces an EPS devoid of Gal (Parniske et al. 1993; Becker et al. 1998). Hence, if USDA 110 and ΔP22 behave as the strain 2143, their growth with Mal as the sole C source should lead to a galactan production in the wild type but an impairment of any EPS production in the mutant.

The amounts of EPS produced by ΔP22 were significantly lower than in USDA 110 under N-sufficiency, in agreement with previous observations referred to above. However, under N-starvation and Mal as the C-source, EPS production was similar in both strains relative either to total protein or to PHB (Table 1), contrary to our expectation of no EPS production in the mutant under these conditions. On the other hand, the accumulation of EPS with respect to PHB followed the same pattern previously observed: in N-sufficiency, EPS was significantly more abundant in young cultures than in old ones, this trend being reversal in the N-starving condition (Table 1). Conversely, PHB accumulation in old cultures was higher

Table 1 Exopolysaccharide production by *B. japonicum* USDA 110 or ΔP22 in different culture conditions

Strain	N condition	Culture age (DAI)	EPS (mg)	
			Protein^{-1} (mg)	PHB^{-1} (mg)
Mal as C-source				
USDA 110	N0	2	2.16 ± 0.22	3.26 ± 0.24
		7	4.84 ± 0.20	12.93 ± 1.51
	N1	2	0.49 ± 0.10	8.24 ± 1.44
		7	0.59 ± 0.11	3.19 ± 0.37
ΔP22	N0	2	1.98 ± 0.15	2.97 ± 0.36
		7	4.28 ± 0.35	13.76 ± 2.32
	N1	2	0.12 ± 0.01	1.98 ± 0.32
		7	0.15 ± 0.01	0.28 ± 0.03
Mtl as C-source				
USDA 110	N0	5	3.16 ± 0.32	13.52 ± 0.65
		14	11.10 ± 0.43	51.61 ± 2.05
	N1	5	0.70 ± 0.24	52.16 ± 3.98
		14	0.27 ± 0.16	0.70 ± 0.03
ΔP22	N0	5	1.57 ± 0.64	8.79 ± 0.30
		14	3.21 ± 0.11	17.73 ± 0.56
	N1	5	0.06 ± 0.03	0.37 ± 0.02
		14	0.09 ± 0.03	0.13 ± 0.04

The amounts of EPS are expressed on the basis of total protein or total PHB for cultures grown with Mal or Mtl as the sole C-source. In each case, the cultures were grown with N-starvation (N0) or N-sufficiency (N1) up to the ages indicated in days after inoculation (DAI), representative of young and old cultures. EPS values given as means ± SE

under N-sufficiency. In 14 DAI Mtl N1 cultures the accumulation of PHB (mg cell protein)$^{-1}$ was 73% higher than in the N-starving condition for USDA 110, while this increase reached 272% in ΔP22. On the other hand, these increases were 19 and 68%, respectively in 7 DAI Mal cultures. Hence, the PHB N-sufficiency/N-starvation increase at stationary phase was, for both C-sources, around 3.5-fold higher in ΔP22 than in USDA 110.

Since the above results did not show differences in EPS accumulation in the mutant strain, relative to the wild type, when cultured in Mal N0 (Table 1), we wanted to determine the EPS composition in both strains, grown in all four culture media, to see whether the change in the C-source or the N-starvation condition could modify it. Purified EPS from 2 DAI Mal or 5 DAI Mtl cultures of each strain in each N condition were hydrolyzed with trifluoroacetic acid and its composition was determined by HPAEC analysis. Different conditions were used in order to quantify neutral and acidic sugars. The wild type USDA 110 strain had a sugar composition of 1 Glu:0.5 Gal:0.5 GalA:0.17 Man, similar to the composition 1 Glu:0.5 Gal:0.5 GalA:0.5 Man already reported by Mort and Bauer (1982), except for the lower Man content (Fig. 2). Differing with the observation made by Karr et al. (2000) for the 2143 strain, the EPS composition in USDA 110 was the same with both C-sources (Fig. 2). As expected, the EPS from ΔP22 lacked Gal (Fig. 2b) but had the same GalA content as the wild type (Fig. 2d). In addition, ΔP22 EPS composition remained unchanged for all the media tested (Fig. 2b, d). The N-condition also had no effect on EPS composition.

Infectivity

To assess the infectivity of these strains we inoculated soybean plants in plastic growth pouches with USDA 110 and ΔP22 grown for 5 DAI in Mtl N1. Twenty days after the plants inoculation, the nodules were visible in the primary roots, even for the ΔP22 strain, which is slower to nodulate (Parniske et al. 1993). Inoculum doses above 10^4 c.f.u. plant^{-1} were required with USDA 110 to obtain more than 80% nodulated pants, but this dose needed to be raised to 10^7 c.f.u. plant^{-1} to obtain the same percent of nodulated plants with ΔP22, thus confirming the low infectivity of this mutant (Parniske et al. 1993).

Previously we observed that *B. japonicum* LP 3001 had higher infectivity when cultured under N-starvation, correlating with its higher EPS content and SBL binding activity in this condition (López-García et al. 2001). Therefore we wondered what kind of response ΔP22 would have, since this strain also produces more EPS under N-starvation than under N-sufficiency (Table 1). Unlike the wild type, this mutant EPS does not bind SBL (van Rhijn et al. 1998) and thus, if this SBL binding were key to infectivity, growth of ΔP22 under N-starvation should have no effect. On the other hand, this strain induces plant defense reactions (Parniske et al. 1994), which are not known if produced by the defective EPS or by another elicitor, masked by wild type EPS. Hence, if the defective polysaccharide were acting as an elicitor, cultures of ΔP22 under N-starvation would have a higher elicitor concentration and therefore, its infectivity should be lower than in N-sufficiency.

We assessed ΔP22 infectivity in plastic growth pouches, using cultures grown for 5 DAI in Mtl N0 or Mtl N1, or 2 DAI in Mal N0 or Mal N1, which were inoculated at 10^7 c.f.u. plant^{-1}. Three soybean plants were sowed per pouch, and RT and SERH positions were marked on the pouches at the moment of inoculation. Twenty days after the plants inoculation, we recorded the infectivity by measuring the distance of each nodule on the primary root to the RT mark. Infectivity of ΔP22 not only did not diminish in the N-starved cultures in relation to N-sufficient ones, but was also higher since more total nodules in primary

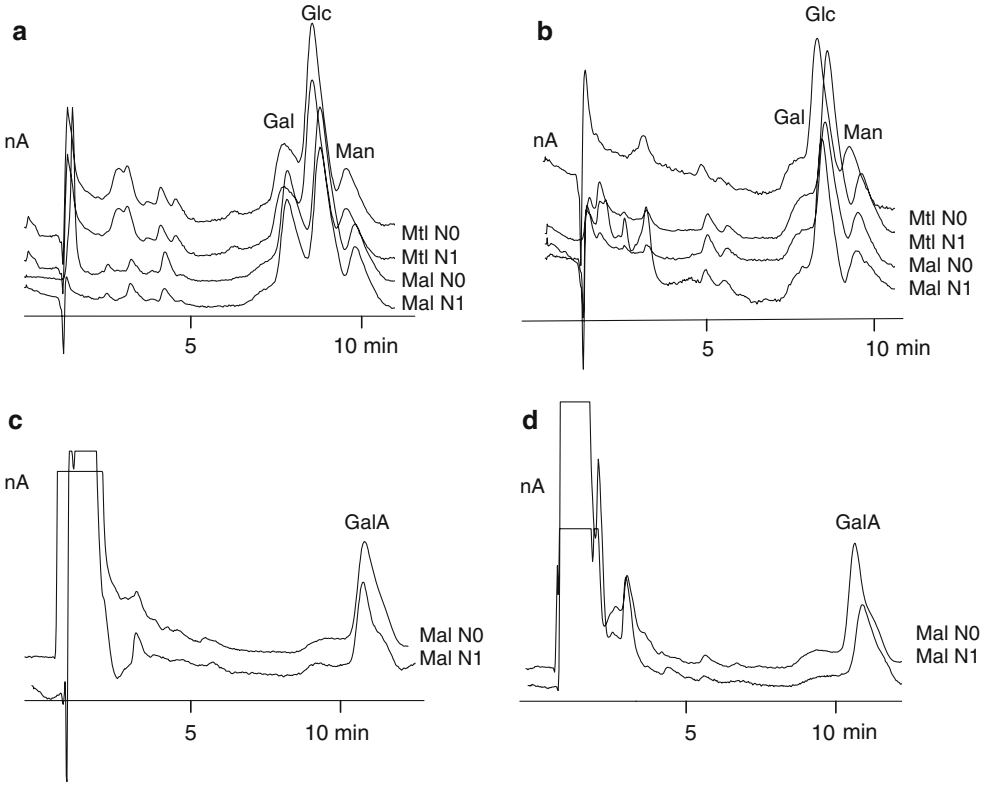

Fig. 2 HPAEC chromatograms for neutral sugars (**a**, **b**) and acid sugars (**c**, **d**) from digested EPS obtained from 2 DAI (Mal) or 5 DAI (Mtl) cultures of *B. japonicum* USDA 110 (**a**, **c**) or ΔP22 (**b**, **d**). The hexoses corresponding to each peak are indicated. For the sake of clarity, in *panels c* and *d* the acid sugars are shown only for cultures grown with Mal as the C-source, since these are at variance with previous reports (Karr et al. 2000); the profiles obtained with Mtl as the C-source were similar, and in agreement with Mort and Bauer (1982). Graph representative of two independent experiments

roots, as well as more nodules above RT mark, were obtained with N-starved cultures irrespective of the C-source. When infectivity was evaluated using as a parameter the mean distance of nodules to RT mark, Mtl-grown ΔP22 rhizobia were revealed as being more infective under N-starvation as this distance was smaller, while Mal-grown cells displayed values similar to the ones obtained in N-sufficient conditions (Table 2). On the other hand, no differences were observed between the C-sources for an equal N-level. Thus, the higher amounts of defective Gal-lacking EPS, produced by ΔP22 under N-starvation, did not reveal any observable impairment in infectivity.

Table 2 Infectivity of *B. japonicum* ΔP22 on soybean roots

Culture medium	Number of nodules in primary root plant^{-1}	Number of nodules above RT mark plant^{-1}	Nodule distance to RT mark (RDU)
Mal N0	2.03 ± 0.34	1.20 ± 0.28	–1.44 ± 0.27
Mal N1	1.43 ± 0.28	0.80 ± 0.14	–1.18 ± 0.28
Mtl N0	2.03 ± 0.32	1.26 ± 0.24	–0.78 ± 0.17
Mtl N1	1.30 ± 0.26	0.77 ± 0.20	–1.17 ± 0.27

The negative values for nodule distance to RT mark (0 RDU) represent root positions below this mark. Values given as means ± SE

Discussion

The ability of free-living *B. japonicum* to grow with only micromolar amounts of N source at the same rate as in a N-sufficient medium (López-García et al. 2001) was confirmed here using two different C- and energy sources, both in a wild type and a mutant strain defective in EPS biosynthesis. In addition, growth rate was higher in both strains with Mal than with Mtl as the sole C- and energy sources, indicating that the rate of energy use was higher with Mal. Total OD_{500} readings obtained with the mutant under N-starvation were consistently higher than with the wild type (Fig. 1). This OD_{500} could be attributed either to the cells or extracellular material, such as EPS. Since the amounts of EPS produced by the mutant strain were equal or less than those corresponding to the wild type, the higher OD_{500} readings obtained with the mutant could indicate a higher total biomass. A lower EPS accumulation in the mutant could result in a more favorable energy balance for biomass production; however, the observed amounts of EPS produced by the wild type and mutant strains under N-starvation were quite similar, especially with Mal as C-source (Table 1). In addition, it remains unclear why the higher biomass was produced only when growth was limited by N. A more careful mass-balance analysis is required to

understand this behavior, but this cannot be obtained with batch cultures as used in our experiments.

Accumulation of EPS in relation to culture age followed a similar trend in the wild type and mutant strains either with Mtl or Mal as C-source. Under N-starvation, net EPS accumulation was observed both in relation to total protein and PHB content (Table 1). By contrast, in the N-sufficient state, the amount of EPS diminished from younger to older cultures, relative to PHB. This was due to the preferred channeling of excess C towards PHB in this condition, as we previously observed with the wild type strain LP 3001 (López-García et al. 2001). Production of PHB in N-sufficient cultures could be related to a fermentative response arising from these conditions (Amarasingham and Davis 1965; Encarnación et al. 1995; Ong and Lin 2003) although the action of general regulators cannot be discarded (Encarnación et al. 2002).

Comparing EPS accumulation in relation with the C-source, there was a higher accumulation with Mtl than with Mal in the wild type strain under N-starvation (Table 1). The higher EPS production could be due to the use of equimolar quantities of each C-source, since half the molar amount of Mtl than that for Mal is required to render the same amounts of activated Glc for EPS synthesis. However, the differences were observed in conditions where the C-source was not limiting and the overflow pathway could be occurring because of energy excess. Nevertheless, this energy would be used more efficiently if EPS biosynthesis starts from Mtl rather than from Mal, where at least four additional ATP moles per mole of activated Glc would be required.

Previous studies with the 2143 strain showed that its EPS composition changed according to the C-source. When Mal was used as the sole C-source, a galactan was observed instead of the well-known *B. japonicum* EPS (Karr et al. 2000). Therefore, it was surprising that the ΔP22 mutant produced similar amounts of EPS as the wild type in Mal N0 since this mutant does not produce UDP-Gal, the only precursor of Gal moieties in EPS (Parniske et al. 1993). Since EPS composition is critical to lectin binding and host recognition (van Rhijn et al. 1998), infectivity (Gage 2004), and plant defense suppression (Niehaus et al. 1997; Fraysse et al. 2003), we considered it important to analyze the composition of this EPS in comparison with the wild type under the conditions tested. We found that, differing from the 2143 strain (Karr et al. 2000), USDA 110 and its derivative did not change their EPS composition with Mal or Mtl as C-sources (Fig. 2), which suggests that changes in EPS composition in response to the kind of C-source are not a general behavior in *B. japonicum*. Similarly, USDA 110 is among the strains that do not produce the NPS upon differentiation to the bacteroid state (Streeter et al. 1992); thus this strain shows a reluctant behavior to change EPS composition in response to environmental stimuli. Further studies are needed to understand the reason why some strains change their EPS composition while others do not, as well as the signal(s) perception and response regulation involved.

Unlike the previously described EPS repetitive unit, our analysis revealed that Man was defective in relation to Glc, Gal, and GalA, with the proportion 6 Glc:3 Gal:3 GalA:1 Man. This composition is consistent with the repeating unit proposed by Mort and Bauer (1982) if one assumes that for every three repeating units, Man is present in only one; the other two being Man-lacking tetrasaccharides. Alternatively, the existence of two EPS could be proposed—for instance one EPS lacking Man and the other as described by Mort and Bauer (1982) in a molar ratio 2:1. Nevertheless, these results, obtained with more sensitive technology, lead to a reconsideration of the standard EPS composition in *B. japonicum* and demonstrate the need for more detailed studies on its structure.

As expected, the EPS from ΔP22 lacked Gal derivatives (Parniske et al. 1993; Becker et al. 1998) but had normal amounts of GalA (Fig. 2). This result suggests that GalA is not incorporated from oxidized UDP-Gal but perhaps comes from oxidation of UDP-Glc and further epimerization of the resultant UDP-glucuronic acid (UDP-GlcA). Two UDP-Glc dehydrogenase and two UDP-GlcA-4′-epimerase ORFs were predicted in the *B. japonicum* genome sequence (RhizoBase designations blr2383, bll8129, blr2382, and bll5920 respectively) although their possible products were not yet characterized (Kaneko et al. 2002). Such enzymes, if active, could catalyze the formation of UDP-GalA from UDP-GlcA (Kereszt et al. 1998; Laus et al. 2004) thereby explaining the presence of GalA residues in the EPS from ΔP22.

As observed earlier, ΔP22 was less infective than USDA 110. We do not know whether this defect is a consequence of the lack of Gal from the mutant EPS—presumably due to the mutation in *exo*B—or to EPS shorter chain length. Previous studies indicate that the size of the EPS produced by ΔP22 is consistent with the expected polysaccharide length for a single repetitive unit (Becker et al. 1998). Studies carried out in *S. meliloti* EPS I (succinoglycan) have shown that single repetitive units are symbiotically inactive, whereas the active forms are dimers and trimers (González et al. 1998). Dimerization and trimerization of the repetitive unit in the succinoglycan is controlled by

*exo*T, and thus, the deletion of this gene in ΔP22 could impair the formation of dimers (Louch and Miller 2001) provided its function is similar to the *S. meliloti* gene. Alternatively, the repetitive units polymerization could not proceed if these are not completed due to a lack of Gal, independently of *exo*T. The deletion in ΔP22 also includes the C-terminal portion of ExoP, which comprises its cytoplasmic domain. Although such a deletion could have an influence on EPS polymerization (Niemeyer and Becker 2001), *B. japonicum* mutants P9 and P23, which had an insertion in the same C-terminal part of ExoP as in ΔP22 but without defects in either *exo*T or *exo*B, were similar to the wild type in general EPS properties and symbiotic behavior (Becker et al. 1998).

Parniske et al. (1994) reported that the diminished infectivity of ΔP22 correlates with a plant defense reaction in the tissues infected by this mutant strain, involving the release of glyceollin and the activation of chitinase activity. Since those experiments were carried out with whole cells, it was not determined whether this plant response was against the defective EPS itself. Several reports from other bacterial species suggest that EPS might function as a suppressor of plant defense reactions triggered by different elicitors (Niehaus et al. 1997) and thus, it is possible that the defects in the EPS synthesized by ΔP22 render this molecule unable to suppress plant defense reactions rather than being an elicitor itself. This is consistent with our observation that under N-limiting conditions, the infectivity of ΔP22 is not less than in N-sufficient media (Table 2). If the defective EPS were the elicitor, then the higher amount of this defective EPS under N-starvation should lead to a stronger defense response and therefore, less infectivity. The absence of Gal, on the other hand, rendered this EPS unable to bind SBL (van Rhijn et al. 1998). Therefore, stimulation of infectivity of the ΔP22 mutant under N-starvation cannot be related to SBL binding to EPS but instead, another condition should explain this behavior. One possibility is that cells approaching stationary phase, as in 2 DAI Mal N0 or 5 DAI Mtl N0, would be intrinsically more infective than exponentially growing cells, as 2 DAI Mal N1 or 5 DAI Mtl N1. But previous studies suggested that early stationary phase rhizobia are not more infective than exponentially growing ones (Bhuvaneswari et al. 1983; López-García et al. 2001). On the other hand, a more sensitive response to genistein for *nodC* gene induction was observed in N-starving *B. japonicum* free-living cells (López-García et al. 2001), which could, in part, explain the higher infectivity of Mtl N0-grown ΔP22 cells in relation with N-sufficient ones.

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